(12) UK Patent Application (19) GB (11) 2 020 424 A

(21) Application No 7914580
(22) Date of filing 26 Apr 1979
(23) Claims filed 26 Apr 1979
(30) Priority data
(31) 903109
(32) 5 May 1978
(33) United States of America (US)
(43) Application published 14 Nov 1979
(51) INT CL²
G01N 33/16
(52) Domestic classification
G1B BA BR
(56) Documents cited
None
(58) Field of search
G1B
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(54) **Determination of glucose**

(57) An improved hexokinase coupled enzyme assay procedure, for measuring the glucose content of body fluids, comprises addition of D-Mannose to the reaction system.

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SPECIFICATION

Kinetic measurement of glucose concentrations in body fluids and formulations for use therein

This invention relates to a novel method and formulation for the kinetic determination of glucose concentration in body fluids using the hexokinase coupled enzyme assay method.

The know method of coupling the enzymes hexokinase and glucose-6-phosphate (G6P) dehydrogenase offers a highly specific system for the determination of glucose in body fluids, such as serum, by reacting the sample containing glucose to completion according to the following enzymatic steps (Adenosine diphosphate is ADP; adenosine triphosphate is ATP; Nicotinamide-adenine-dinucleotide is NAD; reduced NAD is NADH):

(1)

$$\text{D-Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{G6P} + \text{ADP}$$

(2)

$$\text{G6P} + \text{NAD} \xrightarrow{\text{G6P dehydrogenase}} \text{6-phosphogluconate} + \text{NADH}$$

The formation of NADH, measured spectrophoto-metrically is 340 nm, is directly proportional to the glucose concentration in the fluid sample once all the glucose has been reacted.

It has been recognised that a need exists for an assay procedure that eliminates the necessity for completion of the enzyme coupling reaction in order to give greater sample through-put/unit time. It has also been recognised as desirable to eliminate the need for serum blank absorbance corrections which are costly and time-consuming. Moreover, it would be desirable to have a kinetic reaction rate assay procedure which was generally more specific and more easily automated.

It has heretofore been proposed to employ a kinetic fixed time assay for glucose using the hexokinase/glucose-6-phosphate dehydrogenase reagent system employing N-acetyl-glucosamine as a selected reaction kinetic inhibitor.

It has now been found that a suitable coupled enzymatic assay procedure using the hexokinase/glucose-6-phosphate dehydrogenase reagent system can be employed for the kinetic measuring of a body fluid for glucose concentration where the rate of NADH formation is proportional to the rate of glucose reacting, which in turn is proportional to the glucose concentration in the body fluid sample.

Such an improved coupled enzyme assay procedure has been made possible by the finding that the addition of D-mannose alters the kinetic properties of the hexokinase enzyme reaction step. The D-mannose is converted to mannose-6-phosphate *in situ* which acts as a product inhibitor of hexokinase thereby causing the rate of the NADH formation to be proportional to the glucose concentration in the sample.

The resulting system exhibits linear kinetics at about 30°C. for at least one to more than five minutes, depending upon glucose concentration.

It has generally been found that the inclusion of about 4 grams of D-mannose per liter of reagent accomplishes the desired result of providing a system whereby the rate of NADH formation is proportional to the rate of glucose concentration in the fluid sample. Such a kinetic system of glucose determination compares very favorably in accuracy and precision with the heretofore used glucose end-point method. Thus, this kinetic system is adaptable for use on any centrifugal or fast kinetic analyzer, such as, for example, the Gilford 2000 or 3500 models, the Perkin-Elmer KA-150 and other analyzers.

The kinetic reaction system of this invention provides a linear calibration curve over the glucose concentration range of from about 50 to at least about 750 mg/dl and is linear for a period of up to at least 5 minutes over a temperature range of about 23 to 37° C.

The rate at which glucose is reacting is determined by measuring the absorption of NADH spectrophotometrically at about 334 to 366 nm, preferably at 340 nm.

Reagent formulations for the carrying out of the kinetic reaction measurement of glucose concentration according to this invention and depending upon the nature of the clinical instrumentation on which the reagent is meant to be used can be formulated by the inclusion of D-mannose in either a single-vial formulation wherein all the necessary constituents for the test reaction are included in one sample container or in a dual-vial formulation wherein one vial contains a "substrate" containing all reactants except one and a second vial containing the "trigger solution".

Typical examples of such formulations and their preparation are set forth in the following examples:

Example 1
Single Vial Formulation

	Ingredient	*Molecular Weight*	*Specific Amount*	*Range of Amounts*
1.	Add:			
	Dextran	75,000	30 g/l	15-60 g/l
	Bovine Serum Albumin	--	4 g/l	2-8 g/l
	PIPES Buffer Piperazine - N, N'-bis (2-ethanesulfonic acid), monosodium, monohydrate	324	100 mmoles	80-120 mmoles
	Magnesium Chloride, Hexahydrate	203	32 mmoles	30–34 mmoles
	Adenosine-5'-triphosphate, disodium trihydrate (ATP)	605	25.2 mmoles	24.2–26.2 mmoles
	Nicotinamide-adenine-dinucleotide, trihydrate (NAD)	717	3.8 mmoles	2.5–5.0 mmoles
	D-Mannose, anhydrous	180	24.0 mmoles	23.0–25.0 mmoles
	To: 800 ml Deionized Water. Stir until completely dissolved.			
2.	Adjust pH to 7.0 with 50% Sodium Hydroxide Solution at about 30° C.			
3.	Adjust volume to 1 liter with deionized water. Cool mixture to about 1 to 8° C.			
4.	Add:			
	Glucose-6-phosphate dehydrogenase, ex. L. mesenteroides	--	6,000 IU (@ 37°C.)	5,000– 7,000 IU's
	Hexokinase, ex. yeast	--	12,000 IU (@ 37° C.)	10,000–14,000 IU's
5.	Dispense 5.0 ml of solution into 30 cc serum bottles.			
6.	Freeze to about –30° C.			
7.	Lyophilize about 36 to 48 hours with +25° C. shelf heat applied as soon as 200 micron vacuum is reached. Final product temperature not to exceed +30° C.			

8. Stopper and seal under vacuum. Store at 2 to 8°C.

9. Each vial so prepared is to be reconstituted with 20.0 ml distilled or deionized water for use in reaction test system.

Example II

Dual Vial Formulation

	Ingredient	*Molecular Weight*	*Specific Amount*	*Range of Amounts*
1.	For SUBSTRATE, add:			
	Dextran	75,000	30 g/l	15–60 g/l
	Bovine Serum Albumen	--	3.3 g/l	2–8 g/l
	PIPES Buffer Piperazine - N, N'-bis (2-ethanesulfonic acid), monosodium, monohydrate	324	82.5 mmoles	66–99 mmoles
	Magnesium Chloride, Hexahydrate	203	26 mmoles	24–28 mmoles
	Adenosine-5'-triphosphate, disodium trihydrate (ATP)	605	2.2 mmoles	2.1–2.3 mmoles
	Nicotinamide-adenine-dinucleotide, trihydrate (NAD)	717	3.14 mmoles	2.5–5 mmoles
	D-Mannose, anhydrous	180	22.0 mmoles	21.4–22.6 mmoles
	To: 800 ml Deionized Water. Stir until completely dissolved.			
2.	Adjust pH to 7.0 with 50% Sodium Hydroxide Solution at about 30° C.			
3.	Adjust volume to 1 liter with deionized water. Cool mixture to about 1 to 8° C.			
4.	Add:			
	Glucose-6-phosphate dehydrogenase, ex. L. mesenteroides	--	6,000 IU (@ 37°C.)	5,000– 7,000 IU's
	Hexokinase, ex. yeast	--	12,000 IU (@ 37°C.)	10,000–14,000 IU's
5.	Dispense 6.0 ml of solution into 30 cc serum bottles.			

6. Freeze to about −30°C.

7. Lyophilize about 36 to 48 hours with +25°C. shelf heat applied as soon as 200 micron vacuum is reached. Final product temperature not to exceed +30°C.

8. Stopper and seal under vacuum. Store at 2 to 8°C.

9. Each vial so prepared is to be reconstituted with 20.0 ml distilled or deionized water for use in test.

10. For TRIGGER, add:

Dextran	75,000	20 g/l	10−25 g/l
PIPES Buffer	324	16.2 mmoles	14−18 mmoles
ATP	605	172 mmoles	168−176 mmoles

11. Adjust pH to 7.0 (30°C) with 50% Sodium Hydroxide Solution at about 30°C.

12. Adjust volume to 1 liter with deionized water.

13. Dispense 4.0 ml of solution into 15 cc serum bottles.

14. Freeze to about −30°C.

15. Lyophilize about 36 to 48 hours with +25°C. shelf heat applied as soon as 200 micron vacuum is reached. Final product temperature not to exceed +30°C.

16. Stopper and seal under vacuum. Store at 2 to 8°C.

17. Each vial so prepared is to be reconstituted with 11.0 ml distilled or deionized water for use in test.

It will be appreciated that the formulations set forth hereinbefore are merely exemplary and not limited thereto. For example, other suitable constituents may be utilized. In place of the dextran binder there may be

used instead other binders such as gum arabic, mannitol, sorbitol or the like. As other suitable protein sources in place of bovine serum albumin there may be employed, globulins, gelatins or the like. Other suitable buffers include, for example, tris(hydroxymethyl)amino methane, triethanol amine, N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid and the like. Other suitable magnesium salts would include magnesium acetate, nitrate, sulfate and the like.

The usefulness of the formulations of this invention and the linearity of the kinetic reaction rate of the change in absorption of NADH is shown by the following Examples wherein the single vial formulation of Example I was used to measure the change in absorption at 340 nm at 30°C. for standard samples of known glucose concentration. The change in absorption was determined by utilizing a Gilford model 2000 spectrophotometer.

Example III

Assay volume, 2.0 ml reagent + 10 µL sample

Standard Glucose Concentration mg/dl	*Observed rate Δ A 340 nm/min*	*Duration of Linear Rate, minutes*
100	.007	5
200	.014	3
300	.022	3
500	.034	2–1/2
750	.047	2–1/2
1000	.060	2–1/2

Example IV

Assay volume, 2.0 ml reagent + 20 µL sample

Standard Glucose Concentration mg/dl	*Observed rate Δ A 340 nm/min.*	*Duration of Linear Rate, minutes*
100	.014	4
200	.028	3
300	.040	3
500	.065	2–1/2
750	.081	2
1000	.095	2

Example V

Assay volume, 2.0 ml + 25 µL sample

Standard Glucose Concentration, mg/dl	*Observed rate Δ A 340 nm/min.*	*Duration of Linear Rate, minutes*
100	.019	4
200	.035	2–1/2
300	.051	2–1/2
500	.066	2–1/2
750	.086	2–1/2
1000	.102	2

CLAIMS

1. A method for the determination of glucose concentration in a fluid sample by the hexokinase coupled enzyme assay procedure, in which D-mannose is added to the enzyme reaction reagent system, so that the overall reaction rate is substantially proportional to the glucose content of the fluid sample.
2. A method according to claim 1 in which the glucose concentration is determined by measuring the absorption of nicotinamide-adenine-dinucleotide reduced at 340 nm.
3. A method according to claim 1 or claim 2 in which the amount of D-mannose in the reagent system is about 6 millimoles/litre of reagent.
4. A method according to claim 1 substantially as described in Example III.
5. A reagent formulation for the determination of glucose concentration in a fluid sample by the hexokinase coupled enzyme assay procedure, the formulation comprising D-mannose.
6. A formulation according to claim 5 in which the amount of D-mannose is about 6 millimoles/litre of reagent.
7. A formulation according to claim 5 substantially as described in Example I or Example II.

Printed for Her Majesty's Stationery Office, by Croydon Printing Company Limited, Croydon Surrey, 1979
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